Insights from capillary electrophoresis approaches for characterization of monoclonal antibodies and antibody drug conjugates in the period 2016-2018

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ABSTRACT

Monoclonal antibodies (mAbs) and their related products as antibody-drug-conjugates (ADCs) or biosimilars represent a constantly growing class of molecules therapeutic proteins used as treatment against numerous diseases. These compounds can undergo several modifications which could alter the efficiency of treatments. In this context, several analytical methods were designed to deliver a comprehensive structural characterization and guarantee the quality of biotherapeutics. Capillary electrophoresis (CE) is considered today as a major technique for the analysis of biotherapeutics due to benefic characteristics as high resolution separation and miniaturized format. Different CE modes have been developed to characterize mAbs at different levels such as capillary gel electrophoresis (CGE), capillary isoelectric focusing (cIEF), and capillary zone electrophoresis (CZE). Recent developments in CE-mass spectrometry (MS) coupling assessed this technology as a promising tool to obtain high level structural characterization of biopharmaceuticals. Moreover, upcoming techniques such as 2D CE-MS and microfluidic systems are now emerging to offer new possibilities beyond actual limits. This review will be dedicated to discuss the state-of-the-art CE-based methods for the characterization of mAbs and ADCs in the period 2016-2018.

Keywords: Monoclonal antibodies, Biosimilar, Antibody Drug Conjugate, Capillary Gel Electrophoresis, Capillary Zone Electrophoresis, Capillary Isoelectric Focusing, Micro-fluidic capillary electrophoresis, Mass spectrometry, Post-translational modifications

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1. Introduction

Today, more than 75 mAbs and ADCs are approved as therapeutic products by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) [1]. mAbs are occupying the top selling ranks for drugs since last decade [2, 3] making it one of the most successful category of product in the pharmaceutical industry. In addition, the approval of 16 new mAbs over the last couple of years suggests that expansion will further continue [4]. The current technologies to produce recombinant proteins enable to yield different types of biomolecules derived from the mAbs format such as antibody-drug conjugates (ADCs), fusion proteins, bispecific antibodies (bsAb) or biosimilars [5-7]. Their development is currently driven by the treatment of different diseases such as various cancers, infections, autoimmune disorders or to prevent transplant rejections.

Due to their protein nature, these compounds are highly complex macromolecules with typical molecular mass of approximately 150 kDa. However, mAbs can undergo several chemical degradations, post-translational modifications (PTMs) or 3D conformation alterations, which may lead to the inactivation of the drug or immune reactions [8, 9]. These modifications increase the heterogeneity and the complexity of the protein. The characterization and control of protein stability are therefore crucial aspects during each phase of mAbs development, from early-stage development to production, storage, and delivery. For assessment of mAbs and ADCs, regulation agencies have introduced the concept of critical quality attributes (CQA) which describes for each therapeutic protein, the PTMs potentially involved in the alteration of the pharmacokinetic (PK) and pharmacodynamic (PD) properties of the proteins[10, 11]. Thus, stringent analytical methods for comprehensive biotherapeutics characterization are necessary to ensure the quality of this type of products.

Hence, a large array of separation techniques in liquid phase like high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) were developed for quality control of biopharmaceutics [12-15]. These complementary methods allow the separation of the main mAbs isoforms from the modified variants in order to provide a detailed characterization over the different level defining the structure of the protein. In the late 80's, CE has emerged as a powerful technique to analyze biomolecules, thanks to the electromigration phenomenon and the possibility to use aqueous buffers that preserve the high-order structure of proteins [16, 17]. Consequently to the introduction of mAbs, CE has therefore demonstrated to be a relevant analytical separation for the characterization and stability study of this category of protein especially due to the unique selectivity of the electrophoretic separation [14, 18, 19]. Different electrophoretic modes such as capillary gel electrophoresis (CGE), capillary iso-

electric focusing (cIEF) and capillary zone electrophoresis (CZE) are commonly used to assess mAbs. CE experiments are generally performed utilizing optical detection such as UV or fluorescence detector. However, in order to provide structural information, capillary electrophoresis hyphenated to mass spectrometry (CE-MS) has been extensively developed to overcome the limitations of optical detections. Different strategies like intact, middle-up, middle-down and bottom-up analysis were established to access the amino acid sequence and the major PTMs of biotherapeutics [20-22]. Also, CE-MS has been recognized as a powerful tool for immunoglobulin G (IgG) glycan profiling and relative quantitation.

Regardless, the constant introduction of novel mAbs alongside to innovative therapeutic proteins based on this format still maintains the urge to develop further adapted analytical methodologies. Especially, CE has continued to gain a growing interest from the scientific community. This review, following a previous work [14], is focusing on the latest developments regarding the application of CE-based techniques to characterize mAbs and ADCs in the period of 2016-2018. In addition, it provides some insights concerning the future developments for CE based analysis of mAbs and their related formats.

2. Capillary Gel Electrophoresis (CGE)

2.1. Technical considerations

Based on the separation of macromolecules according to their size, CGE represents one of the reference methods to assess size heterogeneity and glycan profiling of mAbs and ADCs. Among CGE techniques, capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) is the adaptation of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) methodology converted to a miniaturized capillary format. That miniaturization involves the replacement of the traditional slab gel by soluble polymers employed as a replaceable molecular sieve. It presents the advantages of short analysis time, to reduce the difficulty of handling, to decrease the amount of sample injected and improve the reproducibility. One major concern of this methodological transfer affecting the separation efficiency, consists of analyte adsorption phenomena in the inner capillary wall. To avoid this harmful phenomena, the development of adapted separation conditions was realized by increasing the concentration and the viscosity of the gel or the inclusion of additives to the BGE. A posivite consequence of the use of additives is the suppression of the electroosmotic flow (EOF). Nowadays, commercial sieving kits are available and widely used in the biopharmaceutical industry. Nevertheless, other strategies have been described to reduce adsorption phenomena and EOF canceling, based particularly on the neutralization of the inner surface of the capillary by chemical reaction. Various commercial or home-made solutions have been reported in the literature as polyvinyl alcohol (PVA) [23] or linear polyacrylamide (LPA) [24], showing the significance of this modification to maintain a good reproducibility of separation.

Detection of analytes in CGE is classically achieved by optical techniques like UV absorption or fluorescence. In most cases, UV detection of proteins is programmed at 220 nm. However, 200, 214 and 280 nm are sometimes used depending on separation conditions. Laser-induced fluorescence (LIF) detection provides an higher sensitivity than classical UV. Generally, the excitation of analytes is performed at 488 nm and the emission signal is monitored at 520 nm. Although, just a few molecules are naturally fluorescent which implies otherwise a derivatization step for the non-fluorescent analytes. Concerning the analysis of mAb glycosylations, chemical modification based on 8-Aminopyrene-1,3,6-trisulfonic-acid (APTS) reaction allows the derivation of fluorophoric group and the addition of three negative charges to the glycan moiety, which is considered today as one of the reference method [23, 25-28]. However recently, other dye reagents like 2-aminobenzoic acid (2-AA) (excitation: 325 nm emission: 405 nm) [29] and Teal™ (excitation and emission: same as APTS) [28], providing respectively one and three negative charges, have been reported for glycan analysis. These methods revealed excellent precision and accuracy. Authors comment about the potential of detecting minor glycan species with Teal™ [28]. Moreover, the Teal™ is preferred for derivatization step because of (i) its higher reactivity, (ii) lower amount of derivatization compound required which contain salts, and (iii) therefore an improved MS compatibility as compared to the other common fluorescent dyes. It can also provide higher sensitivity detection and accurate identification of analytes for CGE-LIF-MS experiments

2.2. Applications

CGE is commonly used for *N*-glycan, size heterogeneity, purity, stability and aggregation assessment of intact and reduced mAbs. ADCs are also characterized by CGE to estimate the drug-to-antibody ratio (DAR). CGE is commonly employed to evaluate the similarity between original glycoproteins and their biosimilar candidates. Table 1 summarizes the applications of CGE for mAbs and ADCs characterization between 2016-2018.

2.2.1. Size variants assessments

Two strategies are established to study mAbs size heterogeneity: non-reduced and reduced conditions. Non-reduced CE-SDS is classically used to control purity/aggregation whereas reduced CE-SDS is mostly used to study mAbs' fragments.

In 2016, Xie *et al.* described a method to analyze the level of reduced disulfide bonds of mAb using non-reduced CE-SDS [30]. They studied the impact of downshift cell culture pH on the formation of this modification. They observed a decrease in the level of reduced disulfide bond by 31 % when lowering pH from 6.95 to 6.75 after 6 days incubation. In another study, Esterman *et al.* evaluated the system suitability acceptance criteria of the U.S. Pharmacopeia (USP) IgG standard using non-reducing CE-SDS and reducing CE-SDS. Two protocols originating from the USP and Bristol-Myers Squibb (BMS) were assessed [31]. Authors concluded that USP method is not suitable

for lot-release and stability testing of mAbs due to a high level of fragmentation under non-reducing conditions. In 2018, Schiel *et al*. analyzed the NISTmAb by non-reduced CE-SDS to assess purity, and reduced CE-SDS to evaluate the glycan occupancies of the heavy chain (HC) alongside to the relative abundances of non-reducible species [32]. Inter and intra-vial homogeneity was evaluated, and excellent standard deviation were calculated (0.053 for intra-vial and 0.0137 for inter-vial). Griaud *et al*. and Miao *et al*. reported two comprehensive studies using an array of orthogonal techniques and strategies including CE-SDS for biosimilarity assessment [33, 34]. They compared the electropherograms of intact and reduced mAbs and their biosimilar candidate. Minor differences were observed but generally the candidate was very close to the originator.

Fragmentation of mAbs represents a CQA that needs to be monitored in order to assess the purity and integrity during the life cycle of the product. Studies of the fragments from stressed mAbs can bring useful information notably to understand mechanism of mAbs degradation [35-37]. These investigations enable to improve mAbs production and storage conditions.

In 2017, Dada *et al*. reported a characterization strategy that establishes the correlation between hinge region fragments analyzed by CE-SDS and size exclusion chromatography (SEC) [38]. Comparative assessment of fragments by SEC, and CE-SDS showed similar correlation with incubation time. Authors analyzed collected fractions of stressed mAbs at different days. An increase of HC, HC-HC aggregates (HH) and HC-HC-LC aggregates (HHL) were observed during incubation. Kubota *et al*. described a CGE method to identify and characterize an impurity of 10 kDa formed after incubation of sample at 25°C for 6 months [35] (Figure 1). In-gel digestion peptide mapping followed by reverse phase liquid chromatography-mass spectrometry (RPLC-MS) and Gelfree 8100 fractionation were used to identify the impurity corresponding to the HC1-104 fragment which is involved in the complementary determining region (CDR). In 2017 , Li *et al*. studied the fragments of an IgG1 obtained by forced degradation at 40°C for 28 days. Two unique fragments were discovered when CE-SDS was employed for the purity analysis of mAbs [36]. Then, these two fragments were fully analyzed and identified to be generated by a Ser105-Ser106 peptide bond cleavage at CDR3 of the HC using different HPLC–MS strategies.

In the 90's, microfluidic transposition of capillary electrophoresis was developed to achieve fast separation for characterization, development, release, and stability testing of therapeutics. Especialy, miniaturization of CGE (mGE) was of great interest for mAbs analysis. Indeed, microchips, due to their intrinsic properties, enable to perform high-throughput analysis while maintaining resolution and efficiency. This device is increasingly recognized as a valuable alternative to conventional CE. In 2016, Cai *et al*. optimized mGE to study mAbs in denaturing conditions [39]. The percentage of SDS was investigated to obtain totally degraded mAb. Three IgG1 and five IgG4 were studied. The reduced mAbs were analyzed by mGE. Electropherograms showed baseline separated peaks of light chains (LC) and HC obtained in less than 30 s. The method could be successfully qualified. The authors demonstrated the precision and the specificity with a linear range of 16-3000 µg/mL and a LOQ of 7.8 µg/mL. Comparison

between mGE and conventional CE-SDS showed that the main difference is the analysis time. Both methods provided similar performances in terms of separation efficiency. However, they noticed that mGE is slightly less sensitive that CE-SDS. In 2017, Smith *et al*. validated a mGE method to study biopharmaceuticals size variants and purity in reducing and non-reducing conditions [40]. Specificity, linearity, accuracy, repeatability, reproducibility, limit of detection/quantification (LOD/LOQ) and robustness were assessed, and results confirmed the excellent features of mGE for reduced and non-reduced mAb analysis.

Concerning the characterization of ADCs, critical information regarding the nature of aggregates and/or fragments can be provided by CE-SDS following different modes of sample preparation as reduced or non-reduced. Nevertheless, this method is more limited for the analysis of ADCs depending of the conjugation chemistry and the attachment sites as lysines, cysteines or glycans [41-44]. Concerning lysine-conjugated ADCs, in 2016, Chen and co-workers published non-reducing CE-SDS reports of trastuzumab emtansine (T-DM1) and the unconjugated trastuzumab. Comparable electropherograms with a main peak corresponding to the full-length antibody and minor peaks representing lower and higher molecular weight species (LMW and HMW) were observed using CE-SDS analysis [42]. The same year, in-depth structural characterization of T-DM1 and its biosimilar candidate were investigated using several orthogonal analytical methods [45]. Characterization of size variants performed with the combination of SEC and CE-SDS exhibited predominant monomer contents (>95 %) in T-DM1 and the biosimilar ADC sample indicating their similarity in the aspect of size heterogeneity. In 2018, Wagh *et al.* reported a comprehensive study in another lysine-conjugated ADCs [43]. Analysis of lysine-conjugated ADCs by non-reducing CE-SDS confirmed a main peak of full-length conjugated antibody and few minor peaks corresponding to LMW and HMW. With reduced CE-SDS, results showed prominent peaks corresponding to HC and LC and minor peaks partially separated for LC with 0 to 2 drug load.

Concerning the interchain-cysteine modified ADCs, CE-SDS profiles look totally different as compared to lysine-conjugated ADCs. Indeed, the conjugation of the cytotoxic drugs to antibodies is performed through cysteine sulfide groups which are activated by a partial reduction of interchain disulfide bonds. It means that some antibody chains are no longer covalently linked by intact disulfide bonds. The presence of SDS in the sample implied the formation of protein-SDS complexes and the breaking of non-covalent interactions. This involves the dissociation of any antibody chains depending on the position and the number of cytotoxic drugs which then causes the presence of low molecular masses ADCs fragments.

2.2.2. Glycan analysis

193 *N*-glycan represents a CQA for biopharmaceutical as it can compromise the efficiency and the safety of mAbs.
194 O'Flaherty *et al*., Zhang *et al*. and Hajba *et al*. reviewed HPLC, CE and MS methods for glycan analysis from mono-
195 saccharides level to intact proteins including *O*-glycans and *N*-glycans until 2016 [46-48]. *N*-linked carbohydrates
196 profiling and quantitation are carried out by CGE. Before analysis, the carbohydrates are first released from mAbs
197 using specific endoglycosidases. Then, glycans are derivatized with a fluorescent dye. Afterwards, labelled glycans
198 are analyzed by CGE-LIF. For the analysis of glycans, some polymers are present in the buffer, but the separation
199 of glycan is not based on a sieving mechanism. The polymer is used to suppress adsorption phenomena and in-
200 crease separation efficiency [49].

201 In 2016 Kovacs *et al*. reported a high-throughput *N*-glycan analysis by CGE-LIF with APTS labeled glycans [25].
202 Using a method named separation window dependent multiple injection (SWDMI) they were able to analyze 96
203 samples in 4h instead of 12h for individual separation cycle. The glycan release step has also been shortened.
204 Szigeti *et al*. developed microcolumns with immobilized PNGase F which carry out rapid and easy to automate *N*-
205 glycan release [26]. This setup allowed them to perform efficient and reproducible deglycosylation of mAb in less
206 than 10 min. More recently, Szarka *et al*. evaluated proton beam irradiation on IgG *N*-glycan to anticipate the effect
207 of a long space travel [50]. After analysis of labeled glycan by CGE-LIF, they concluded that changes occur only
208 above 10 000 Gy which is quite inferior to Galactic Cosmic Ray and Solar Particle Events. Kubo *et al*. reported a
209 new polyethyleneglycol dimethacrylate (PEGDMA) coating [51]. Different conditions for polymerization procedure
210 were optimized. They showed the effective separation of sugars released from mAbs using this coating. Authors
211 suggested that the concentration and ethylene oxide units of PEGDMA affected the range of separable molecular
212 weight.

213 In 2018, Chen *et al*. reported the use of CGE-LIF as an orthogonal method to confirm the *N*-glycan quantitative
214 results obtained by miniaturized CE-MS (mCE-MS) [27]. A DNA analyzer was used for CGE-LIF experiment. Similar
215 results, in terms of quantitation and resolution were obtained but mCE-MS provided faster separation. Quantita-
216 tion of glycans has been successfully done by Szigeti *et al.* [52] over an extended concentration range. In this work,
217 quantitation of Man5 on adalimumab has been investigated. They obtained an excellent (R^2=0.9995) between
218 peak area of Man5 and concentration within a range 0.01-0.5 ng/µL. Authors suggested this quantitation method
219 could be easily applied to other carbohydrates moieties. To further improve the identification of glycosylation
220 patterns, CE-LIF-MS coupling has been developed by Khan *et al*. [28]. This setup allowed the authors to quantify
221 and identify *N*-glycans of NISTmAb (Figure 2). The novel fluorescent dye used is Teal™ which is MS compatible and
222 provides a higher quantum yield than classic APTS. This derivative agent permits to reach qualitative and quanti-
223 tative sugars information. Results of the baseline separations of different *N*-glycans showed excellent correlation
224 between LIF and MS.

225

3. Capillary Isoelectric focusing (cIEF) and imagedcIEF (icIEF)

3.1. Technical considerations

cIEF represents one of the most resolutive methods to separate proteins depending on their isoelectric point (p*I*) [53]. cIEF is the well-known miniaturized analogue of IEF slab gel mainly described for the analysis of charge heterogeneity of mAbs and ADCs [54-58]. Historically, the conventional protocol of cIEF follows a two steps procedure starting by a focusing then a mobilization step. Briefly, cIEF experiment is performed by using a alkaline catholyte (NaOH) and an acid anolyte (H_3PO_4). The capillary is filled with a solution containing carrier ampholytes and analytes. By applying a high voltage, a gradient of pH is established through the whole capillary and proteins are focusing in the capillary until they reach their own p*I*. At this point, the mobility of proteins is near zero because their apparent charge is neutral. Then, sample is forced to migrate toward the detector, which is placed close to the capillary exit using an electrophoretic or hydrodynamic mobilization step. However, the mobilization step can induce some undesired effects like a decrease of resolution or modification of migration time. To address these drawbacks, whole-column imaging cIEF (icIEF) has been developed. In icIEF experiments, UV-detection is performed by a charge coupled device (CCD) camera all along the capillary (5cm) allowing to cancel the mobilization step and achieve faster separation with greater resolution, better reproducibility and reduced sample volume. Commonly to cIEF and icIEF experiments, coating of inner surface capillaries is mandatory to eliminate EOF and improve separation efficiency. For this purpose, neutral capillaries using dynamic or permanent coating have been implemented. In cIEF, the most described coatings are LPA [54, 57, 59], hydroxypropyl methylcellulose (HPMC) [60, 61] and PVA [62]. Concerning icIEF, a majority of reports in the literature described the use of a fluorocarbon capillary due to manufacturer's near monopoly (Proteinsimple) [55, 63-65]. However, other manufacturer like CEInfinite provide icIEF system allowing the use of a wider choice of coated capillary solution.

Over the last couple of years, the technical advances of cIEF and icIEF detection modes have been mainly achieved using coupling with MS. Indeed, concerning UV-detection, almost every application described in cIEF and icIEF are performed at 280 nm due to good absorption of proteins at this wavelength and the strong absorbance of ampholytes at lower wavelengths. MS detection represents a key feature for future developments to improve the performance of cIEF and icIEF in terms of sensitivity and structural characterization [66-68]. However, development of the hyphenation between cIEF and MS is curbed by technical issues such as presence of salts and ampholytes. Very recently, different strategies of cIEF-MS and icIEF-MS coupling have been described in order to reduce or eliminate these adverse effects. In 2017, Huhner *et al.* developed an cIEF-MS methodology using an innovative two dimensional CE-MS instrumentation allowing to perform cIEF or icIEF separation in the first dimension followed by CZE in the second dimension, with online coupling to high-resolution MS [69, 70]. In 2018, Dai *et al.* described a new automated cIEF-MS method to separate mAb charge variants using an electrokinetically

pumped sheath liquid nanospray CE-MS technology [67]. These last developments showed the potential of cIEF-MS coupling to become a method of interest in biopharmaceutical industries and will be deeply discussed in the section 5.

3.2. Applications

cIEF and icIEF play important roles in the characterization of purity and isoelectric point (p*I*) of mAbs. These methodologies are considered as reference methods to determine mAbs charge heterogeneity. Several reports described these techniques for the period until 2016 [14, 15, 37, 71]. In recent years, new enzymatic digestion, new matrix and fractionation method were reported. Table 1 displays cIEF and icIEF applications for mAbs analysis in 2016-2018.

Several inter-laboratory studies to evaluate the robustness of cIEF and icIEF have been detailed these last decades [57, 58]. The most recent in 2018, described by Wu *et al.*, reported an interlaboratory method validation of icIEF for mAbs charge heterogeneity analysis [72]. 10 laboratories and 8 companies were involved. icIEF protocol was performed according to the international conference on harmonization (ICH) guidelines. The method was evaluated on four mAbs. Results confirmed the good precision in p*I* determination, the satisfying separation of charge variants and the high precision for suitable assessment of purity. Following icIEF method, the p*Is* and the charge variants of 23 therapeutic mAbs have been reported by Goyon *et al*.[73]. Experimental p*Is*, determined by icIEF, were ranged between 6.1 and 9.4. Theoretical and measured p*Is* were compared giving absolute deviations always lower than 15%. The relative distributions of acidic and basic variants were also determined to range between 15 to 30 % using both cation exchange chromatography (CEX) and icIEF which was in agreement with the literature. King *et al*. analyzed an IgG1 by hydrophobic interaction chromatography (HIC) and obtained good separation of two peaks [74]. The two peaks were isolated and further characterized by HPLC-MS and icIEF using different enzymatic strategies. p*I* of 7.6 and 7.8 were respectively found for these peaks. Deamidation of an asparagine (deaN) residue, located in the light chain CDR3, has been highlighted. They also showed the negative impact of this PTM for the antigen binding affinity of the mAb. Finally, influence of pH on the deamidation formation was studied. The results confirmed an increasing amount of deamidation at slightly basic pH.

cIEF and icIEF are extensively used to study fragments from degraded mAbs and biosimilarity assessment. Xie *et al*. studied the effect of pH shift on the production of IgG1 mAb in Chinese hamster ovary cells [30]. The p*I* and absolute percentages of acidic, main and basic variants were determined by cIEF. They concluded that a pH downshift reduces the amount of acidic variants and then prevents the formation of PTMs. Further investigations through tryptic peptide mapping using MS highlighted that deamination proportion of HC-Asn388 and HC-Asn394 or HC-Asn393 decreases while pH is lowered. In the same year, Zhang *et al*. developed a icIEF to analyze mAb

fragments yielded by an optimized SpeB digestion (cysteine protease) [75]. With this new strategy, they obtained well resolved peaks of LC, Fd, Fc/2 fragments and their PTMs. They also carried out a study of degraded mAbs and they evaluated the biosimilarity of rituximab and a candidate with SpeB proteolysis. Authors demonstrated the partial removal of C-terminal lysine between the reference and the biosimilar rituximab as well as a lower level of deamidation. The same year, biosimilarity assessment studies on Avastin (bevacizumab) has been reported by Zhao *et al.* [76]. Several analytical methods, including icIEF, were used to compare pharmacokinetics properties of Avastin and a biosimilar (Figure 3). Firstly, mAbs were prepared to obtain isolated acidic, basic and main variants. Then, these fragments were analyzed by different HPLC and CE techniques. Results showed no significant differences in pharmacokinetics parameters. In-depth study of the biosimilarity of tocilizumab candidate was performed by Miao *et al.* [34]. Using different methods such as icIEF, CEX, SEC and RPLC-MS to determine biological and physicochemical characterization, authors illustrated the very high similarity of the biosimilar and the originator tocilizumab.

Classic denaturant like urea or formamide, used in icIEF to stabilize the molecule charge can sometimes deteriorate proteins by aggregation process. Thus, a novel matrix formula for icIEF experiment in native conditions was explored and optimized by Zhang *et al*. to study charge heterogeneity of proteins [77]. This matrix, composed by non-detergent sulfobetaine and taurine (NDSB-T) possesses the stabilization and separation power while maintaining protein integrity. NDSB-T can separate and quantify protein charge species in native state and therefore avoid partial denaturation. This matrix enables to improve the assay robustness, repeatability, precision, accuracy and peak resolution for an aggregation-prone mAb. Another issue for cIEF analysis is the relatively low amount of sample injected which does not permit the fractionation of variants for further characterization. Hosken *et al*. proposed an optimized preparative IEF based technique which allows fractionating the charge variants of intact mAb [78]. They carried out an isoelectric focusing using a free-flow electrophoresis (FFE). FFE consists of two plates between a carrier ampholyte enabling the focusing of charge variants when a high voltage is applied. High amount of sample can be injected and then the isolation of acidic, basic and main variants becomes possible. This method was applied on 3 mAbs. Charge variants of two mAbs were highly enriched except for the variants of the mAb with the highest p*I* which did not focus enough. The enriched fractions were sufficient for physico-chemical and biological characterization and many PTMs were identified. Using another strategy, developers from CEinfinite proposed new methodologies for icIEF coupled to MS and/or peak fractionation. They developed a specific proprietary cartridge specially designed for high performance preparative icIEF using a 200 µm ID capillary followed by a 50 µm ID transfer capillary. This geometry allowed great flexibility such as protein fractionation enabling direct spotting process to MALDI target plate.

In 2018, Turner *et al*. optimized cIEF method for NISTmAb qualification [79]. Separation of basic variants, main peak and acidic variants has been performed and C-terminal lysine has been identified. The authors also realized

the titration of each variant in a range from 0.1 to 0.6 mg/mL with $R^2 > 0.98$ and RSD < 8%. A comparison with an optimized CZE method allowed authors to confirm that cIEF assay is a valuable characterization tool which affords information about charge variant apparent p*I* while CZE assay was found to be suitable for qualification as a routine quality monitoring assay.

Focusing on ADCs analysis, isoelectric focusing methods may give significantly different information on electrophoretic profiles of ADCs [41, 42, 44]. The nature of the drug-linker, especially the charge that can be added to the ADC represents a major concern on the charge profile of the protein. For example, Ji *et al.* described the characterization of thio-succinimide hydrolysis of monomethyl auristatin E (vc-MMAE) ADC using icIEF during the formulation development [80]. As uncharged vc-MMAE conjugation through sulfhydryl groups in the interchain-cysteine residues is known not to change the net charge of the ADCs, similar charge variant profiles were expected between the conjugated and the unconjugated mAb. However, overlay of icIEF vc-MMAE ADC's profiles, incubated at pH 9 during 0 to 48 hours, showed an important heterogeneity in the acidic region due to various negative charges of the ADC gained from both deamidation and succinimide hydrolysis of the thio-succinimide linker [80]. Concerning lysine-linked ADC, this type of chemistry eliminates basic sites in the proteins and changes ADCs p*I*s [81]. Lin *et al.* described the determination of mAb-Drug Maytansinoid 4 (DM4) with an average DAR of 3.6 by icIEF [81]. p*I* of the drug loaded species shifted toward acidic variants from the unconjugated mAb due to the increase in the number of DM4 drugs conjugated. More recently, Luo *et al.* confirmed these results performing the structural characterization of a mAb-DM1 ADCs using the combination of UV/vis spectroscopy, HPLC/TOF-MS and icIEF analytical methods [82]. Average DAR value (3.2) measured with icIEF was in good agreement with UV/vis and intact mass values (3.3 and 3.1 respectively). In 2018, Wagh *et al.* reported that icIEF can be applied to measure the level of unconjugated antibody and drug load distribution, but it cannot distinguish between conjugates, process intermediates and impurities such as antibody with linker only and antibody with linker/drug conjugates. Indeed, conjugation of linker only and linker/with drug bring the same shift charge toward acidic, and therefore cannot be distinguished by icIEF. [43].

4. Capillary Zone Electrophoresis (CZE)

4.1. Technical considerations

Until the first development of CZE theory, this electrophoretic mode is still the most popular for CE applications. This technique leans on a separation of analytes in a background electrolyte (BGE) under an electrical field according to their charge-to-size ratio [16]. Typically, the capillary is filled up with the BGE. After a sample loading in the capillary via a hydrodynamic or an electrokinetic injection, a separation voltage is applied to permit the migration of analytes into the capillary. The most widespread detection mode is UV detection using a single wavelength

detection (usually 214 nm) or a diode array detection. Besides UV, other modes are used in CZE as LIF and more recently MS. CZE-MS coupling requires some optimizations of separation conditions because of the weak tolerance to salts and the need of volatile solvents. Once these parameters laid down, MS detection enhances structural characterization of the samples. A complete section about the CE-MS coupling will be dedicated in the section 5 of this review.

Concerning the characterization of mAbs and ADCs, as CE-SDS and cIEF modes, the main issue of the CZE technique was the protein adsorption on the capillary wall. Especially for middle-up and intact mAbs analysis, dynamic or permanent chemical modification of the inner surface of the capillary must be performed prior to CZE separation. The majority of detailed protocols in the literature concerns the use of a dynamic HPMC coated capillary which is considered as a reference for mAb charge variants analysis [14, 83, 84]. However, different strategies have already been described with other dynamic positive coating as Polybrene [85] or neutral coating as polyethylene oxide [86, 87]. More recently, in 2017, Xiao *et al*. have developed an innovative coating inspired by a well-known biological phenomenon, the blood coagulation, and worked on a fibrin coating on an open tubular column [88]. Apart from this parameter, the optimization of experimental conditions concerning the mAb analysis in CZE depends enormously on the physicochemical properties of the mAbs to characterize them at the desired levels and will be described in the application section 4.2.

4.2. Applications

4.2.1. Bottom-up analysis

At the bottom-up level, major concerns regarding mAbs characterization such as glycoprofiling, isomerizations and amino-acid modifications, have been solved with CZE methods. Feng *et al*. have highlighted the *N*-glycosylation of two human IgGs with a comparison of CZE and micellar electrokinetic capillary chromatography (MEKC) [23]. They worked on a PVA coated capillary to obtain a separation of 26 glycoforms referenced in the national institute for bioprocessing research and training (NIBRT) glycobase in 17 minutes in CZE and 24 min in MEKC. They confirmed their results with additional tests combining α-fucosidase, α-neuraminidase and ß-galactosidase. To further improve mAbs characterization, Griaud's team studied ranibizumab (Lucentis®) and a biosimilar (razumab®) by SEC, CE-SDS and CZE methods. After optimization, they separated and identified multiple serine to asparagine sequence variation sites on the mAbs. They checked the results with a MS detection and exposed a difference of 27.01 Da (6-9 %). The analysis of MS/MS spectra confirmed serine to asparagine substitution on the light chains [33]. The same year, disulfide isomers were separated with a high resolution. The study was focused on the development of an ultra-high voltage capillary electrophoresis (UHVCE) and the analysis of 3 mAbs and 1 ADC. The analysis has been made with a 120 kV electric field (2 kV/cm) in a HPMC coated capillary. This system

enhanced resolution and separation speed allowing distinction of disulfide isomers [89]. More recently, CZE has also been developed to get the best repeatability in particular with one of the key source of differentiability of digestion. An integrated micro reactor has been set up to perform an in-line tryptic digestion to enhance the quality control of mAbs. After an off-line optimization step of enzyme concentration and incubation time, other major parameters have been adjusted in-line to determine the best nature of BGE (ionic strength, pH). The feasibility and robustness of the project have been evaluated on three commercial mAbs: trastuzumab (Hercpetin®), infliximab (Remicade®) and tocilizumab (Roactemra®). The diffusion mediated proteolysis combined with an electrophoretic separation (D-PES) has been developed on a polyethyleneoxide (PEO) coated capillary regenerated before each analysis. A specific injection pattern of mAb, trypsin and proteolytic buffer has been elaborated allowing to produce a digest with the same efficiency as compared to the off-line protocol. This totally automated method is clearly a promising solution decreasing the total analysis time by a factor of four (5 hours needed to digest, separate and analyze a sample), decreasing the volume of reactants by 1000 fold and offering a great repeatability of the total ion electropherograms (TIE) [90].

4.2.2. Intact analysis

One of the highest challenges of separation methods dwells in intact proteins analysis. While CZE is not considered as a reference method so far, the development of this approach showed significant advances for mAb charge variants analysis. Today, a classical CZE protocol from the pioneer works of He *et al.* [91, 92] which is evaluated by an interlaboratory study [93], can be described as the use of a BGE composed of ε-aminocaproic acid (EACA), triethylenetetramine (TETA) and HPMC. In 2017, a complete tutorial about a quite similar protocol, has been published in video format illustrated the separation of mAbs' isoforms on a neutral capillary. These favorable conditions increase efficiency, accuracy and repeatability while keeping undamaged capillary up to 150 injections [94]. Illustrating complexity to optimize the large number of parameters, Suba *et al*. described a "two-phase-four-step" approach allowing a rapid, iterative method development process for CZE [95]. Based on fluorocarbon coated capillaries, they set up a fast screening phase to adjust the pH and the ionic strength with the aim to get optimal migration time, stability and resolving capacity of the method. Then, a fine tuning phase leaned on the addition of TETA to enhance the viscosity and improve the peak resolving and the stability of the method. They concluded that optimal conditions were 50 mM EACA at pH 5.1 with 1 mM TETA and 0.05% v/v HPMC. The same year, Zhao *et al*. detailed the characterization of a biosimilar from Bevacizumab (Avastin®) using different methods as SEC, icIEF, CEX and CZE [96]. They indicated a purity of the acidic, basic and main peaks respectively of 94 %, 82 % and 85 %. In addition, PK of this candidate has been studied showing that all the results are acceptable to validate it as a biosimilar. However, PK and purity are not the only parameters required to allow the commercialization of

this kind of molecules. Additional tests with other analytical methods need to be employed to enable the characterization at different levels. Moritz *et al*. described a design of experiments (DoE) based on differences between the net charge and the hydrodynamic radius to optimize charge heterogeneity tests [97]. The DoE set up during this study highlighted important parameters for CZE optimization. Described work is promising for a successful development of CZE as a major method for biopharmaceuticals analysis. Alternative approach consisting of exploring new BGE conditions for mAb charge variants separation, has also been developed by Goyon *et al*. in 2018 [87]. 17 marketed mAbs have been evaluated using solely a Bis-Tris buffer (pH 7.0) and PEO dynamic coating. Goyon *et al*. obtained a RSD < 5 % of migration times and the separation of acidic charge variants of 10 / 17 mAbs. They also compared their methodology with the reference CZE method involving EACA, TETA and HPMC. The novel CZE approach allowed separating additional charge variants (in particular more acidic variants). Conversely, a higher number of basic variants were separated by the reference CZE approach for two mAbs out of 17, demonstrating the complementarity of the two approaches. The relative quantitation of acidic and basic variants has been established specifically 15-30 % of acidic variants and 5-15 % of basic variants (Figure 4). Authors concluded that their method used to separate native mAbs offers complementary results but extend five folds the analysis time and elicits a loss of sensitivity. The same year, Kahle *et al*. realized a comparison of protein charge variants separated by CZE and cIEF to upgrade the quality control of batches [59]. Another approach on the development of innovative coating was performed by Xiao *et al*. They reported an easy-to-use coating which gave a good separation of cetuximab (Erbitux®) and rituximab (Mabthera®) with respectively nine and five separated variants [88]. By contrast, in a bovine serum albumin (BSA) coated column, they distinguished seven and four variants for the same molecules. These separations were detected by UV detection at 280 nm and gave a satisfying repeatability with a deviation times < 2.42 % on day to day and run to run batches.

To assess the robustness and the reproducibility of CZE methods, Kubota *et al*. proposed a validation study of CZE method for evaluating mAbs and ADCs [84]. Among the numerous tested parameters in this study, are represented identity, purity, specificity, linearity, precision, quantitation limit, repeatability, accuracy, range and robustness of CZE to characterize biopharmaceuticals. 11 samples including 2 ADCs and 9 mAbs have been evaluated in this study with different p*I*, DAR, and structures. The method showed quantitative results with high specificity, separation efficiency and precision. Authors concluded that CZE appears to be a promising alternative to IEF and CEX. The nearest validation step has been established by Schiel *et al*. in 2018. In two separate studies, they investigated the reference material (RM 8671) called NIST mAb which is a first-of-kind reference material to inform on specific attributes during therapeutic proteins characterization [32, 79]. The identity, quality, stability of the NIST mAb have been studied. The CZE assay was found to be suitable for qualification as a routine quality monitoring assay due to excellent sensitivity, speed, simplicity, specificity, and intermediate precision. Based on their results, CZE was selected for longitudinal quality monitoring of the NISTmAb Reference Material charge heterogeneity.

5. Capillary electrophoresis – mass spectrometry (CE-MS)

5.1. Technical considerations

The growing need of precise characterization of biotherapeutics implies the constant evolution in separation methods especially with the achievement of additional structural information. As the performance of mass spectrometers improved in term of sensitivity, mass accuracy and scanning frequencies, MS became a key instrumentation for the analysis of biomolecules. The complexity due to the large scale of heterogeneity on mAbs needs to take the sensitivity into account to characterize low amount isoforms. While the different miniaturized electrophoretic modes (CE-SDS, cIEF, CZE) coupled with spectrophotometric detection are already known to be reference methods for mAbs characterization, the main improvements developed these last few years were achieved in the development of CE-MS methods. Since the end of the eighties, instrumental developments have been constantly performed in order to optimized the hyphenation of CE with MS detection using offline or online approaches depending on the nature of the MS sources: electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) [98]. CE-MS coupling is usually implemented with ESI; however, specific CE-MALDI-MS applications were also described in the literature [99-101]. As the weak acceptance of salts and the need of volatile solvents is known to be primary parameters for CE-MS applications, one of the main issue for CE-MS instrumentation is known to conserve a continuous electric field during CE separation while maintaining a stable spray. This last decades, a way to get around this issue has been to design innovative CE-MS interface geometries. Historical developed CE-MS interfacing systems relied on a conductive sheath-liquid, connected to the outlet electrode of the CE system, continuously injected to maintain the electrical field [102]. Despite a good robustness and an easy setup, regarding the very low amount of sample carried in the separation capillary, the dilution effect due to the sheath-liquid has a negative impact on MS sensitifity. To address this limitation, other geometries have been developed allowing to reduce to the minimum the flow rate or to wholly eliminate the sheath-liquid. Today some commercial CE-MS solutions based on sheath liquid and sheathless interface are already fully described in the literature and commercially available [98]. Nevertheless, regarding direct CE-ESI-MS, the main hurdle quite in common of all papers detailed in the literature consisted of the incompatibility of classical well known BGEs with MS detection due to the presence of nonvolatile compounds that diminishes drastically the quality of MS data. While some very recent articles describing improvement of CZE-ESI-MS or cIEF-ESI-MS applications for mAbs charge variants characterization [67, 103, 104], another recent approach have been developed to reduce BGE impact on CE-ESI-MS detection. Inspired by 2D LC-MS, the group of Prof. Neusüβ worked on a promising 2D CE-MS setup, pursuing to provide interference-free mass spectra with electrophoretic separations [105]. This advanced separation technique includes a first dimension which allows to perform a high resolving CE separation with nonvolatile BGEs

485 followed by a second dimension able to fully remove the ESI-interfering components from the analytes prior to
486 MS detection. For this purpose, a mechanical four-port valve with a small volume loop was utilized to lead the
487 analytes from the first dimension to the second one with limited diffusion. The main advantage of this approach
488 is the possibility to perform the 1st dimension in any CE mode (CZE, cIEF, CE-SDS) with classical conditions discussed
489 in previous sections (BGE, coating, additives, etc.). This type of instrumentation has the potential to further in-
490 crease the level of characterization achieved in a single experiment, but still needs some technical improvements
491 to make it robust and get a marketed solution.
492
493 **5.2. Applications**
494 **5.2.1. Amino acid sequence characterization**
495 MAbs are produced from a biosynthesis process in culture cells from living organisms that could induce some
496 minor differences in the amino acid sequence or PTMs during the maturation phase of the proteins. Moreover,
497 production, packaging and storage conditions of the molecules can also induce some amino acid substitutions or
498 other sort of modifications. Since 2006, Gennaro *et al.* demonstrated the potential of CZE-ESI-MS to obtained the
499 complete characterization of mAbs using a Lys-C digestion combined with HPLC-MS analysis [106]. In 2013, Ga-
500 houal *et al*. confirmed the positive impact of sheathless CZE-ESI-MS methods demonstrating the consistent char-
501 acterization of the amino acid sequence with 100% coverage and some PTMs such as *N*-glycosylations, *N*-terminal
502 glutamic acid cyclization, asparagine deamidations (deaN), methionine oxidations (oxyM), and aspartic acid isom-
503 erization (isoD) in a single analysis [107, 108]. In 2016, Said *et al*. adapted the CZE-ESI-MS method, especially with
504 the optimization of the tryptic digestion protocol, for the characterization of the primary structure of a cysteine-
505 linked ADC. Results revealed the method ability to characterize ADCs primary structure particularly with the loca-
506 tion of drug loaded peptides and the estimation of their conjugation level [109]. More recently, some aque-
507 ous/aprotic dipolar solvent mixes systems were tested by Dada *et al*. to enhance the peptide mapping on mAbs
508 and ADCs. Authors explained that N,N-dimethylacetamide (DMA) and N,N-dimethylformamide (DMF) used as BGE
509 allow to have a complete sequence coverage and improve the separation of peptides with a neutral coated capil-
510 lary at the expense of time analysis [110]. The results confirmed several advantages of CZE-ESI-MS for the analysis
511 of small hydrophilic di-/tripeptides, large hydrophobic peptides, glycopeptides, and hydrophobic drug-linked pep-
512 tides.
513
514 **5.2.2. Glycosylation characterization**
515 Glycosylation is a PTM that occurs naturally during excretion of antibodies from the expression system to the
516 extracellular medium. It only represents 2–5% of the total mass of the protein but it is subjected to extensive

studies due to its significant influence on effector functions of mAbs [111, 112]. As a consequence, the mAbs glycosylation profile is considered as a CQA and must be thoroughly analyzed [113-115]. For over 10 years, numbers of analytical methods have been developed to better understand the synthesis, roles and consequences of mAbs glycosylations [111, 116, 117]. The last development of CE-MS methods highlighted the attractiveness to characterize and quantify the glycosylation heterogeneity with high accuracy and precision [107]. In 2016, a quantitative twoplex glycan analysis using heavy and light carbon stable isotopes of 2-aminobenzoic acid (2-AA) labelling have been developed to check the quality of different mAbs batches [118]. Glycosylation profiles have been detected and identified with a fluorescence detector on different cetuximab batches. Comparison with orthogonal techniques as HPLC-MS were realized using a released glycan approach. Results showed a good agreement and the reproducibility of the two methods. In 2017, Giorgetti *et al*. evaluated the CZE-ESI-MS method to perform relative quantitation of *N*-glycan species for mAbs characterization at the peptides level [119]. Validation in terms of robustness and reproducibility were demonstrated through the relative quantitation of glycosylation profiles for ten different mAbs and two biosimilar produced in different cell lines. A systematic comparison was performed with hydrophilic interaction liquid chromatography (HILIC) reference method, obtaining very similar glycosylation pattern. Some glycan differences can also be provided from a difference of linkage between two glycosylation units. Sialic acid linkages differences have been observed by Kammeijer *et al*. using CZE-ESI-MS [120]. Although identical fragmentation patterns obtained by collision induced dissociation (CID), authors detailed the selective analysis of α 2,3 and α 2,6 –sialylated glycopeptides with no additional sample preparation. The different migration behavior between the two moieties was found to correlate with differences of pKa values which is consistent with the selectivity of the separation. In 2018, Chen *et al.* worked on the glycoprofiling of the NIST mAb with a mCE-MS interface [27]. Authors proposed a quick solution for intact mAb characterization with quantitative results on protein variants. They obtained a partial separation and the relative quantitation of 18 variants including some glycolytic modifications. In the last few months of 2018, Jooβ *et al.* described a method based on CZE coupled to drift tube ion mobility MS for the analysis of native and APTS-labeled *N*-glycans [121]. In their study, each individual glycan signal separated in CZE exhibited an unexpected high number of peaks observed in the IMS dimension. Among the possible explanations, the type of sialic acid attached to glycans has a significant impact on the IMS heat map.

5.2.3. Middle-up analysis

Biopharmaceutics analysis at the bottom-up level involves sample treatments as digestion protocol which is known to potentially induce some artefactual modifications of amino acids implying possible over estimation of PTMs. To avoid or reduce these limitations, optimizations of sample preparation were developed to study mAbs

at other levels such as middle-up or intact ones. Middle-up analysis consists of a prior partial and localized mAbs digestion by the mean of specific enzymes and reduction reagents. Obtained fragments, usually larger than 10 kDa, gave the opportunity to focus on specific parts of the mAbs structure with a lower difficulty than intact molecules. The middle-up level has been made possible due to the development of proteolytic enzymes like *Streptococcal endopeptidase* (IdeS) or papain. On contrary to bottom-up level, middle-up strategies allow to enhance the sample preparation protocol by reducing the number of chemical steps and then minimizing modifications induced by digestion.

Due to the limited proteolysis of mAbs which generates significantly larger peptides as compared to classical trypsin or Lys-C digestions, different strategies of online and offline CE-MS coupling have been developed in the last ten years. Biacchi *et al.* detailed an offline CE-MALDI/ESI-MS method for the analysis of Fc/2 and F(ab')2 fragments produced from the IdeS digestion of cetuximab [122]. They concluded on the baseline separation of Fc/2 lysine variants and the characterization of the *N*-glycosylation sites present on the mAb. In 2016, the same group deepened their study by demonstrating the separation in native conditions of three Fc/2 homo- or heterodimers depending on the presence of a C-terminal lysine [123]. The first study using an online CE-ESI-MS coupling for mAbs separation at middle-up level was described by Han *et al.* in 2016 [124]. Authors demonstrated the baseline separation of HC and LC after reduction of disulfide bridges as well as the complete separation of Fc/2, Fd and LC fragments after Ides digestion followed by a reduction step. Concerning IdeS digestion, they obtained in less than 20 minutes, a separation of Fc/2 lysine variants and observed mass differences between control, deamidated and oxidized stressed mAbs.

Very recently, two major articles were published on the multi-level characterization of diverse mAbs combining middle-up and intact strategies using CZE-ESI-MS methods. Belov and coworkers developed CZE-ESI-MS methods to study different IgG1 at the intermediate level with an IdeS digestion but no reduction step [104]. CE conditions consisted of the use of a 1-(4-iodobutyl) 4-aza-1-azoniabicyclo[2,2,2] octane iodide (M7C4I) positively coated capillary with a BGE composed of 50 % ethanol added to 1 % formic acid and a voltage of 20 kV. They obtained a separation of Fc/2 and F(ab)'2 fragments in less than 60 minutes, with the characterization of modifications such as oxidations and deamidations. In addition, they studied the same samples with a combination of IdeS digestion and a TCEP reduction step. They obtained separation of respectively LC, Fc/2 and Fd fragments in nearly 40 minutes. Both experiments allowed to characterize different glycoforms on the Fc/2 fragments. The same year, Somsen's team succeed in separating Fc/2 glycoforms by varying BGE [125]. Neutral LPA capillary has been employed due to the near zero EOF generated during the separation and to avoid protein adsorption on the capillary wall. They discussed the effect of acetic acid concentration as BGE. A complete characterization of the different Fc/2 variants has been detailed with the glycan and C-terminal Lys variability and the observation of other PTMs as deamidation.

583

584 **5.2.4. Intact protein analysis**

585

586 Analysis of intact protein, which is considered particularly challenging, represents the absolute way of protein
587 characterization. Indeed, due to the reduction or even the absence of sample preparation which can introduce
588 artefactual modifications, intact protein analysis has the advantage to be more representative of the actual sam-
589 ple. However, due to the high masses and the complexity of mAbs, intact level analysis of these therapeutics is
590 still a challenge for analytical sciences. CE-MS coupling has been described as a promising technique for the char-
591 acterization of intact proteins [126] and then has been applied particularly these last years in the field of biother-
592 apeutics [14]. In 2014, Biacchi *et al*. detailed the first separation of intact mAbs using an offline CZE-MALDI-MS
593 coupling [100]. The offline property of the CE-MS coupling allows the authors to work with salt-concentrated and
594 non-volatile BGE enabling them to obtain the separation and MS analysis of intact charge variants of trastuzumab.
595 Unfortunately, limitation of MALDI-MS resolution for molecules up to 100 kDa did not allow the measurement of
596 the exact mass of these charge variants. Direct CE-ESI-MS coupling then appears as a solution to avoid this limita-
597 tion. In 2016, Han *et al.* proposed the separation and identification of free LC contained in IgG1 reference material
598 using CZE-ESI-MS method [124]. The same year, Ramsey's group developed and employed a CE-nanoESI-MS mi-
599 crochip for the analysis of intact infliximab [127]. Molecular weights were determined for five charge variants
600 separated on a 23 cm long separation channel coated with an aminopropylsilane (APS) base layer and covalent
601 modification with polyethylene glycol (PEG). Three major species corresponding to C-terminal lysine variants plus
602 some minor acidic and basic species have been characterized with an average resolution of 0.80. The same group
603 used this method for the characterization of a lysine-linked ADC [128]. Authors observed the separation of five
604 main species that differed in their respective DAR, which for each DAR displayed the same variant population
605 observed on the unconjugated mAb. They concluded that the CE-nanoESI-MS microchip method demonstrated
606 the possibility of accurately determine the DAR.

607 In 2017, Belov *et al.* have been the first group to analyze intact mAb by online native CZE-ESI-MS [22]. Working
608 on a sheathless CE-MS interface coupled with an orbitrap mass spectrometer and using neutral polyacrylamide
609 coated capillary, they obtained a partial separation of intact mAbs aggregates. Native condition as 20 mM ammo-
610 nium acetate pH 8.0 allowed them to reveal predominant forms of the intact mAb, corresponding to 2X-glycosyl-
611 ated, 1X-glycosylated and dimeric structures. They also observed other molecular species of about 101 kDa, pre-
612 sumably corresponding to the mAb with the loss of 2 light chains. The dissociated light chains have been separated
613 as monomeric and dimeric forms. In 2018, the same group worked on another mAb under denaturing conditions
614 and using a positively charged coating capillary (M7C4I) [104]. Optimized BGE condition composed of 10% isopro-
615 panol, 0.2% formic acid allows them to report the complete glycosylation profile of the mAb. Three types of mAb

glycans populations (2X-glycosylated, 1X-glycosylated and aglycosylated forms) have been observed (Figure 5). Very soon after this paper, Haselberg *et al.* performed the heterogeneity assessment of intact trastuzumab and ustekinumab using sheathless CE-MS interface coupled with a Q-TOF MS instrument [125]. For the two mAbs, partial separation of charge variants has been obtained using 10% acetic acid BGE and a neutral capillary coating consisting of a hydrophobic layer to protect siloxanes from hydrolysis, and a second layer of polyacrylamide providing a hydrophilic surface. Each peak appeared to consist of variants mixtures with different properties depending of the nature of the mAb. The same year, a third paper about intact mAb separation using sheathless CZE-ESI-MS has been published by Giorgetti *et al.* [103]. Using 3% acetic acid BGE, sample buffer of 30% methanol 1% formic acid and a PEI positively coated capillary, separation of three world-wide health authorities approved mAbs (rituximab, palivizumab and trastuzumab), have been evaluated. CZE-ESI-MS analysis of these three mAbs showed partial separation obtained in less than 20 min allowing identification of mAbs isoforms. For each mAb, 2X-glycosylated and 1X-glycosylated structures have been identified and separated. Concerning basic and acidic variants, minor differences between 0 and 2 Da have been observed suggesting potential iso-Asp modification and deaN. However, mass accuracy of the mass spectrometer added to separation performances, did not allow to conclude without ambiguity on the nature of these modification.

As a well-known property, MS-based methods present the main drawback to be intolerant to classical salted and non-volatile BGE reducing the possibility of optimizing CE methods. Indeed, in a comparison between reference CZE-UV and CZE-ESI-MS methods using different BGE, obtained electropherograms fitted well but lower resolutions were obtained for CZE-ESI-MS [103]. In 2017, Jooβ *et al.* described a CZE-CZE-MS method for the characterization of intact mAbs charge variants [129]. Authors achieved interference-free, highly precise mass data (deviation less than 1 Da) of trastuzumab charge variants. The mass accuracy obtained (< ppm) were discussed regarding both measured and calculated masses. The same group confirmed the advantages of their 2D CE-MS system with the development of a cIEF-CZE-MS for the characterization of intact mAbs [69]. After focusing step, the analytes were mobilized through two external detectors (UV and C^4D) to select precisely analytes inside the transfer loop and then to run the CZE dimension prior to MS detection. They established a "multiple heart-cut" approach considerably reducing the analysis time allowing them to perform the transfer of up to six analytes from a single cIEF run and the analysis via CZE-MS in less than 3h. Application on the MS characterization of intact mAb charge variants enabled them to detect a mass difference of approximately 2 Da between two variants. In 2018, the same group deepened the development of the 2D CE-MS coupling to identify low abundance charge variants of trastuzumab using icIEF-CZE-MS [70]. icIEF first dimension can be monitored in real-time thanks to the whole-column detection. Accurate masses were attributed to deamidation, formation of succinimide or cyclisation of *N*-terminal glutamic acid for the main acidic and basic variants. Despite the difficulty to achieve online cIEF-ESI-MS application due to CE experimental condition, Dai *et al.* reported a new online cIEF-ESI-MS method for mAb charge

variant analysis using an electrokinetically pumped sheath-flow nanospray ion source with pressure-assisted chemical mobilization [67]. Many critical parameters and reagents have been optimized including MS-friendly anolyte and catholyte, a glycerol enhanced sample mixture or ampholyte. Charge variant profiles of trastuzumab, bevacizumab, infliximab and cetuximab, obtained using cIEF-MS method, were corroborated by icIEF-UV analysis. Moreover, charge variants of these mAbs were characterized using the online intact MS data. More recently, the same group applied their cIEF-ESI-MS method for the in-depth characterization of cetuximab charge heterogeneity [68].

6. Conclusion

Due to the inherent structural complexity of the mAbs format, a wide variety of analytical techniques, including liquid chromatography, electrophoresis and mass spectrometry have been implemented. Detailed characterizations of these therapeutic proteins have been obtained by these techniques to ensure the integrity of the drugs. Therefore, the physicochemical phenomenon involved in CE separation demonstrated in numerous applications a unique benefit for mAbs analysis. Conventional CE separation modes like CZE, CE-SDS or (i)cIEF are particularly relevant to characterize mAbs size and charge variants thanks to CE intrinsic features which provide efficient and highly-resolutive separations. Furthermore, the different inter-laboratory studies support unambiguously the confidence of the analysis in addition to the robustness provided by the CE-based analytical methods. As a consequence, CE has gradually become one of the reference technique in the biopharmaceutical industry to assess purity and stability of mAbs. Concomitantly, CE-MS hyphenation was implemented and demonstrated to be relevant to access precious structural information like amino acid sequences, glycosylations, PTMs and higher-order structures.

In the most recent period, the development of analytical methodologies involving CE separation has not diminished, which is emphasized by the significant number of articles discussed throughout the present review. Also, it is important to note that each CE separation mode is currently explored for the development of methods dedicated to the characterization of mAbs and their related formats. Such interest is indeed attributed to an improved understanding of the charge based selectivity of the electrophoretic separation in the case of mAbs, which showed to be relevant to distinguish several types of micro-heterogeneities. In addition, the introduction of innovative molecules based on the mAbs format like ADCs contributes to the dynamic of CE method development.

The implementation of CE-ESI-MS has also benefiting further from various instrumental progresses. Regarding that aspect, the recent emergence of 2D CE-MS represents a significant breakthrough. Indeed, 2D CE-MS enables to potentially improve separation and peak capacity in a similar manner as 2D liquid chromatography. The intro-

duction of microfluidic technologies has also exhibited the possibility to achieve short-time analyses while maintaining a high resolution due to the reduction of diffusion effects, for instance to perform a high-throughput screening of mAbs and ADCs.

Future prospects regarding CE based analysis of mAbs and their associated formats suggest CE-MS hold the most important potential due to the complete orthogonality of both techniques and the performances of current generation of high resolution MS instruments. Also, by performing CZE using a MS compatible BGE over the second dimension, 2D CE-MS enables to perform separation mode previously incompatible with MS coupling like CE-SDS or cIEF over the first dimension. That aspect of 2D CE-MS is particularly promising because it enriches in a drastic manner the applicability of CE-MS, therefore further developments is excepted in that direction in the near future. For instance, it is possible to envisage the use of specific CE analysis like affinity CE. The application of affinity CE in conjunction with MS is particularly appealing for the characterization of mAbs. During their lifetime, mAbs are interacting with targeted antigens in addition to several types proteins which influence their effector functions and/or pharmacological properties. Thus, by distinguishing protein-protein/protein-ligand complexes and determine affinity constants using their respective electrophoretic mobilities, it could be possible to correlate structural modifications with mAbs affinity opening the path to structure-function analysis. The development of affinity CE-MS experiments adapted to mAbs represents a major challenge however the introduction of 2D CE-MS clearly reshuffles the cards on that aspect. Finally, because native MS analysis is often limited by the absence of separation before the MS analysis, the development of CE-MS analysis performed could improve significantly the sensitivity and widening the applicability of native MS. This could be applied especially for the analysis of complex mixtures while maintaining therapeutic mAbs is a state as close as possible to the protein administrated to the patient.

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Caption

Fig. 1. Electropherograms of the mAb-A initial sample (lower trace) and degradation sample (upper trace) obtained by CE-SDS (a) reduced and (b) non-reduced conditions. The internal standard peak, light chain peak, heavy chain peak, monomer peak, and increased peaks are indicated. Especially, the increased peak close to the internal standard is the focus of this study. Reprinted from [35]. Copyright (2017) Elsevier.

Fig. 2. Online CE-LIF-MS analysis of Teal™-labeled NISTmAb released *N*-glycans. LIF and MS base peak electropherogram (m/z 620-1,800) of Teal-NISTmAb glycans using ammonium hydroxide based BGE. Reprinted from [28]. Copyright (2017) Wiley.

Fig. 3. Imaged capillary isoelectric focusing for profile of the isolated charge variants, the biosimilar product and Avastin. Reprinted from [76].

Fig. 4. Charge profiles of a mixture comprising valid bevacizumab, trastuzumab and rituximab solutions (blue trace) and expired solutions (orange trace). Reprinted from [87]. Copyright (2018) Wiley

Fig. 5. Deconvolution spectrums showing major glycosylation states of the intact mAb. Quantitative ratios of each population of the mAb (2X-glycosylated, 1X-glycosylated, aglycosylated) are specified. Reprinted from [104]. Copyright (2018) Wiley.

Table 1. Application and CE methods for the analysis of IgG, biosimilars, Fc-fusion proteins and ADCs in the years 2016-2018

Abbreviation: PBS phosphate buffer saline; EACA ε-amino caproic acid; TETA triethylenetetramine; BFS bare fused silica; LPA linear polyacrylamide; PVA polyvinyl alcohol; HPMC hydroxypropyl-methyl cellulose; HPC hydroxypropyl cellulose; FC fluorocarbon; PB polybren; DS dextran sulfate; PEO Polyethylene oxide; APS aminopropylsilane; APTES aminopropyltriethoxysilane

CE Mode	Detection	Sample	Application	BGE	Cap. coating	Reference
CE-SDS mGE	UV 220 nm	mAb	Size heterogeneity of reduced mAb	SDS gel separation buffer	BFS	[39]
CE-SDS	UV 220 nm	Ado-trastuzumab emtansine & biosimilar	Biosimilarity assessment, size heterogenetiy of intact and reduced mAb	SDS gel separation buffer	BFS	[45]
CE-SDS	UV 220 nm	IgG1	Disulfide bond reduction	SDS-MW gel buffer	BFS	[30]
CE-SDS	UV 220 nm	IgGs	Purity and stability assessment	SDS-MW gel buffer	BFS	[31]
mGE		mAb	Intra and extracellular evaluation of LC:HC ratio			[130]
CE-SDS	LIF 488 nm 520 nm	IgG	High-throughput N-glycan analysis	NCHO Carbohydrate Separation Buffer	NCHO separation capillary	[25]
CE-SDS	LIF	IgG	N-glycan analysis	1% PEO in 25 mM lithium acetate (pH 4.75)	NCHO separation capillary	[26]
mGE	Indirect LIF	bsAb	Size heterogeneity of reduced and intact mAb	HT Protein Express Sample Buffer		[131]
CE-SDS	UV 220 nm	Recombinant IgG1	Size heterogeneity of intact and reduced mAb	SDS-MW gel buffer	BFS	[38]
CE-SDS	LIF 488 nm 520 nm	Ranibizumab and biosimilar (labeled)	Biosimilarity assessment, size heterogeneity of intact mAb	0,1 M Tris-HCl/5% SDS pH 8	BFS	[33]
CE-SDS	LIF 488 nm 520 nm	mAbs	Glycan profiling	89 mM aqueous Tris-boric solution pH 8.64	PEGDMA or PAA	[51]
CE-SDS	UV 220 nm	mAb A	Size heterogeneity of reduced and intact mAb	SDS gel separation buffer (AB Sciex)	BFS	[35]
CE-SDS	UV 220 nm	Recombinant IgG1	Quantitation of impurety from degraded mAb	SDS sieving gel buffer (Beckman)	BFS	[36]
CE-SDS	UV 220 nm	IgG1	Size heterogeneity of reduced and intact mAb	SDS-MW gel buffer	BFS	[38]

CE-SDS	UV 220 nm	Tocilizumab & biosimilar	Biosimilarity assessment, size heterogeneity of reduced and intact mAb	SDS sieving gel buffer (Beckman)	BFS	[34]
CE-SDS	UV 220 nm	NIST mAb	Fab fragment characterization	SDS-MW gel buffer	BFS	[132]
CE-SDS	LIF 488 nm 520 nm	hIgG	N-glycan characterization	1% HPC (w/v), 1% HEC (w/v), 80 mM MES,40 mM TRIS	PVA	[23]
CE-SDS	LIF 325 nm 405 nm	Rituximab, Trastuzumab & Bevecazumab	N-glycan analysis	100 mM Tris-borate buffer (pH 8.3) 5% PEG	DB-1	[29]
CE-SDS		Lys-conjugated ADC	Size heterogeneity of reduced and intact ADC			[43]
CE-SDS	LIF	hIgG	N-glycan characterization of irradiated mAb	N-CHO	BFS	[50]
CE-SDS	LIF	NISTmAb RM 8671	Glycan profiling	POP 7 polymer		[27]
CE-SDS-LIF-MS	LIF 488 nm 520 nm, LTQ-Orbitrap XL	NISTmAb RM 8671	Glycan analysis	NH4OH or AcNH4	BFS	[28]
CE-SDS mGE	LIF 488 nm 520 nm	Fc fragment of adalimumab	Glycan absolute quantification	HR-NCHO	BFS	[52]
icIEF	UV 280 nm	mAb-DM1 ADCs	Drug Load Distribution and DAR determination		FC	[82]
icIEF	UV 280 nm	3 recombinant hmAb	Charge heterogeneity of intact mAb, comparison with FFE	Amph : 3-10 Anol 80 mM H3PO4 Cath : 100 mM NaOH	Coated capillary from Convergent Bioscience	[78]
cIEF	UV 280 nm	IgG1	Charge heterogeneity, structural characterization	Amph : 3-10 Anol 200 mM H3PO4 Cath : 300 mM NaOH	eCAP™ Neutral	[30]
icIEF	UV 280 nm	Rituximab & biosimilar	Charge heterogeneity of intact mAb and mAb's fragments	Amph : 2% 5-8 & 8-10.5 Anol : 0,08 M H3PO4 Cath 0.1 M	FC	[75]

				NaOH + 0,1% MC in both		
icIEF	UV 280 nm	IgG1	Charge heterogeneity, purity assessment	Amph : 3-10 : 8-10.5 (1:1)		[76]
icIEF	UV 280 nm	23 marketed mAbs	Charge heterogeneity, pI determination	Amph : 3-10 Anol : 80 mM H3PO4 Cath : 100 mM NaOH + 0,1% MC in both	FC	[73]
icIEF	UV 280 nm	Tocilizumab & biosimilar	Charge heterogeneity of intact mAb	Amph : 3-10		[34]
icIEF	UV 280 nm	Antibody A	Charge heterogeneity of native mAb	Amph : 3-10		[77]
icIEF	UV 280 nm	IgG1, IgG2 & IgG4	Interlaboratory study Charge heterogeneity of intact mAb, purity assessment	Amph : 3-10 + 1%MC	FC	[72]
icIEF	UV 280 nm	IgG1	Charge heterogeneity of intact mAb	Anol : acid Cath : base		[74]
cIEF-MS icIEF-UV	UV 280 nm &TOF	Infliximab, Trastuzumab, Bevacizumab & Cetuximab	Charge heterogeneity of intact mAb	cIEF-MS : Amph : 3-10 Anol : 1% formic acid Cath : 0,2 N NH4OH + 15 % glycerol in both iCIEF-UV : Amph : 3-10 Anol : 80 mM H3PO4 Cath : NaOH + 0,1% MC in both	cIEF-MS : Neutral coating PS1 iCIEF-UV : FC	[67]
cIEF CZE	UV 280 nm	NISTmAb RM 8671	Charge heterogeneity	Amph : 3-10 : 8-10.5 (1:3) Anol 200 mM H3PO4 Cath : 300 mM NaOH	Neutral coating (Sciex PN 477441)	[79]

CZE icIEF	UV 214 nm	mAbs	Characterization of mAb variants	40 mM EACA; 2 mM TETA pH 6.0	HPMC	[59]
CZE	UV 214 nm	mAb ADC	Method validation	380 mM EACA, 1.9 mM TETA, pH 5.7 0.05% HPMC	HPMC	[84]
CZE		mAb	Method development for mAbs quality	50 mM EACA; 1 mM TETA pH 4.1	HPMC	[95]
CZE	UV 280 nm	Bevacizumab and bio-simlar	Characterization of mAb charge variants	500 mM EACA	BFS	[96]
CZE	214 nm	mAb	Characterization of mAb isoforms	200 mM EACA; 30 mM lithium acetate	HPMC	[94]
CZE CE-SDS		Human IgG	Glycosylation profiling	25 mM NH4Ac, pH 4,75	PVA	[23]
CZE CE-SDS	UV 214 nm	Ranibizumab; Ra-zumab (biosimilar)	Serine to Asparagine sequence variation	400 mM EACA; 2 mM TETA pH 5.7	BFS	[33]
CZE	UV 280 nm	mAb; ADC	Charge variants and disulfide iso-mers of mAbs and ADCs	400 mM EACA; 2 mM TETA pH 5.7	HPMC	[89]
CZE	UV 214 nm	mAbs	Charge heterogeneity of mAbs	40 mM EACA; 2 mM TETA pH 6.0	HPC; HPMC	[97]
CZE	UV 280 nm	Cetuximab, Trastu-zumab, Rituximab	Characterization of mAb variants	50 mM Phosphate buffer	Fibrin	[88]
CZE	UV 214 nm	17 marketed mAbs	Characterization of mAb variants	200 mM BisTris pH 7.0	PEO; HPMC	[87]
CZE	UV 280 nm	mAbs	In-line tryptic digestion of mAbs automation	25 mM EACA; citric acid pH 5.0	PEO	[90]

CZE	UV 214 nm	NISTmAb	NISTmAb homogeneity value assignment and stability	400 mM EACA; 2 mM TETA pH 5.7; 0.03% Tween 20	HPMC	[32]
CZE	UV 214 nm	NISTmAb	Charge heterogeneity of mAbs	400 mM EACA; 2 mM TETA pH 5.7; 0.03% Tween 20	HPMC	[133]
mCE-ESI-MS	ESI-TOF-MS	IgG1, IgG2, ADC, Infliximab	Characterization of mAb variants	100 mM PBS + 10 g/L NHS-PEG	APS	[127]
CZE-MS	MALDI-TOF-MS	Cetuximab	Fc/2 dimers characterization	Inlet vial: 200 mM EACA/25 mM $C_2H_7NO_2$ Outlet vial: 25 mM $C_2H_7NO_2$	HPC	[123]
CZE-MS	ESI-QTOF-MS/MS	Trastuzumab	Asparagine deamidation and iso Aspartic acid isomerization	10% CH_3CO_2H	BFS	[108]
CZE-MS	ESI-TOF-MS	IgG1	Charge heterogeneity of reduced mAb Middle up analysis	10% - 30% CH_3CO_2H SL: 50% CH_4O/0.5% COOH	LPA	[124]
mCE-ESI-MS	ESI-QTOF-MS	ADC	Characterization of intact ADC	10% C_3H_8O + 0.2% CH_3CO_2H	PEG, APS	[128]
CZE-MS	ESI-QTOF-MS/MS	Brentuximab vedotin ADC	Intact, middle-up and bottom-up characterization	10% CH_3CO_2H	BFS	[109]
CZE-MS	ESI-QTOF-MS/MS	Cetuximab	Glycan analysis	25 mM $C_2H_7NO_2$	PVA	[118]
CZE-MS	ESI-Orbitrap-MS/MS	standards proteins *E. Coli* ribosomal proteins trastuzumab	Proteins, protein complexes and organellar proteomes analysis	40 mM $C_2H_7NO_2$	BFS PA	[22]
CZE-MS	MALDI-TOF-MS	Cetuximab	MAb Fc/2 charge variants analysis	Inlet vial: 200 mM EACA/25 mM $C_2H_7NO_2$ Outlet vial: 25 mM $C_2H_7NO_2$	HPC	[21]

CZE-MS	ESI-Orbitrap-MS/MS	ADC	Peptide mapping of therapeutic proteins	20% CH_3CO_2H 15% DMA 1% *m*-nitro-benzyl alcohol	LPA	[110]
CZE-MS	ESI-Orbitrap-MS/MS	mAb	Improvment peptide mapping of therapeutic proteins	20% CH_3CO_2H/15% DMA or DMF	LPA	[134]
CZE-MS	ESI-QTOF-MS/MS	Rituximab; palivizumab; natalizumab; nivolumab; trastuzumab; panitumumab; adalimumab; inflximab-remicade; inflximab-remsima; inflximab inflectra	Mabs N-glycosylation profiling	10% CH_3CO_2H	BFS	[119]
CZE-MS	ESI-QTOF-MS/MS	Human polyclonal IgG recombinant monoclonal IgG1	Sialic acid linkage differenciation of glycopeptides	10% CH_3CO_2H	BFS	[120]
CZE-MS	ESI-QTOF-MS/MS	IgG1	Eliminate SDS interferences in antibody analysis	1 M CH_3CO_2H SL: C_3H_8O/0.2% CH_2O_2 (50:50)	PVA PB	[135]
CZE-MS	ESI-Orbitrap-MS/MS	mAb	Analysis of host cell impurities in mAbs	2 M CH_3CO_2H SL: 10% CH_3OH/0.5% CH_2O_2	LPA	[136]
CZE-MS	ESI-Orbitrap-MS/MS	IgG1	Middle-down and intact mAb proteoform characterization	50% CH_3OH/1% CH_2O_2 10% C_3H_8O/0.2% CH_2O_2	M7C4I PA	[104]
mCE-ESI-MS	ESI-Orbitrap-MS/MS	NIST mAb	NIST mAb proteoforms and glycoforms characterization	0.2% CH_3CO_2H/10% C_3H_8O	APS	[27]

cIEF-MS	ESI-TOF-MS	Infliximab, trastuzumab, cetuximab, bevacizumab	Intact mAb charge variants analysis	Amph: 3-10 + 5-20% glycerol Anol: 1% CH_2O_2 + 15% glycerol Cath: 0.2% NH_4OH + 15% glycerol	Neutral PS1	[67]
cIEF-MS	ESI-TOF-MS	Cetuximab	Intact and middle-up characterization	Amph: 3-10 + 5-20% glycerol Anol: 1% CH_2O_2 + 15% glycerol Cath: 0.2% NH_4OH + 15% glycerol	Neutral PS1	[68]
CZE-MS	ESI-QTOF-MS	Infliximab, trastuzumab, ustekinumab	Heretogeneity assessment of antibody derived therapeutics	10% CH_3CO_2H	Neutral	[125]
CZE-MS	ESI-QTOF-MS	Trastuzumab, rituximab, palivizumab	Intact mAb charge variants analysis	3% CH_3CO_2H	PEI	[103]
CZE-MS	ESI-Orbitrap-MS/MS	mAb	Glycosylation profiling	$C_2H_7NO_2$ SL: 10 mM $C_2H_7NO_2/C_2H_3N$ (20/80)	BFS	[28]
CZE-MS	IMS-Q-TOF-MS	mAb	Glycosylation profiling	0.1 M EACA, 0.5 M NH_3 solved in MeOH/H_2O 1:1	BFS	[121]
cIEF-CZE-MS	UV; ESI Q-TOF-MS	Deglycosylated mAb	Charge heterogeneity	cIEF :Amph : 3-10 ou 6-8 Anol H3PO4 Cath NaOH CZE : 0,2 M HCOOH	PVA both	[69]
CZE-CZE-MS	UV 214 nm ; ESI Q-TOF-MS	Trastuzumab	Intact mAb charge variants	CZE 1 : 380 mM EACA, 1,9 mM TEA and 0,05% w/w HPMC (pH	CZE 1 : BFS CZE 2 : PVA	[129]

				5.7) CZE 2 : 2 M acetic acid		
cIEF-CZE-MS	UV 280 nm; ESI-Q-TOF-MS	Trastuzumab	Charge heterogeneity	icIEF : Amph : AESlyte, Anol : 80 mM H3PO4, Cath : 80 mM NaOH 0.1 % MC both CZE : 1M acetic acid	PVA both	[70]

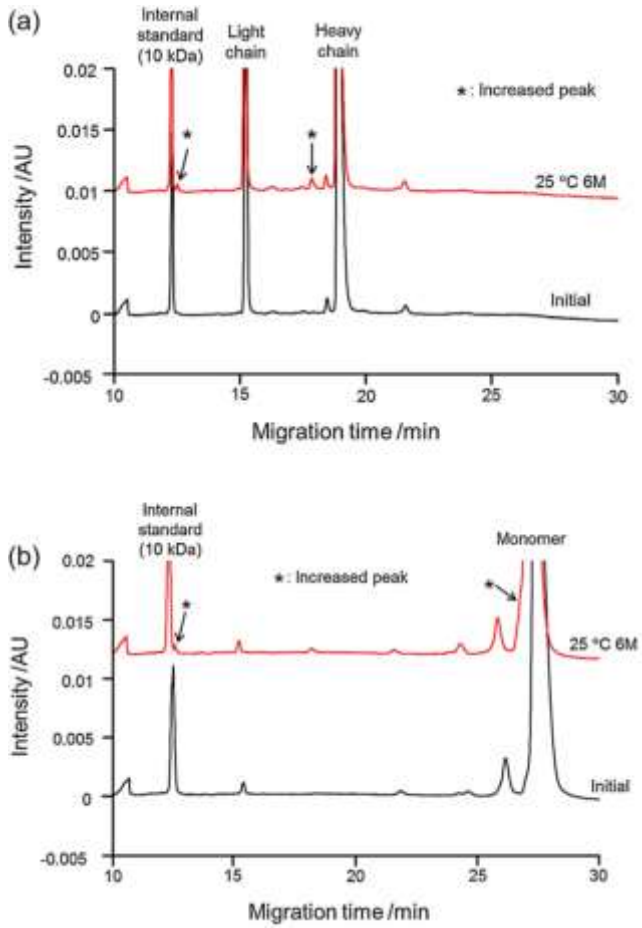


Fig. 1. Electropherograms of the mAb-A initial sample (lower trace) and degradation sample (upper trace) obtained by CE-SDS (a) reduced and (b) non-reduced conditions. The internal standard peak, light chain peak, heavy chain peak, monomer peak, and increased peaks are indicated. Especially, the increased peak close to the internal standard is the focus of this study. Reprinted from [35]. Copyright (2017) Elsevier.

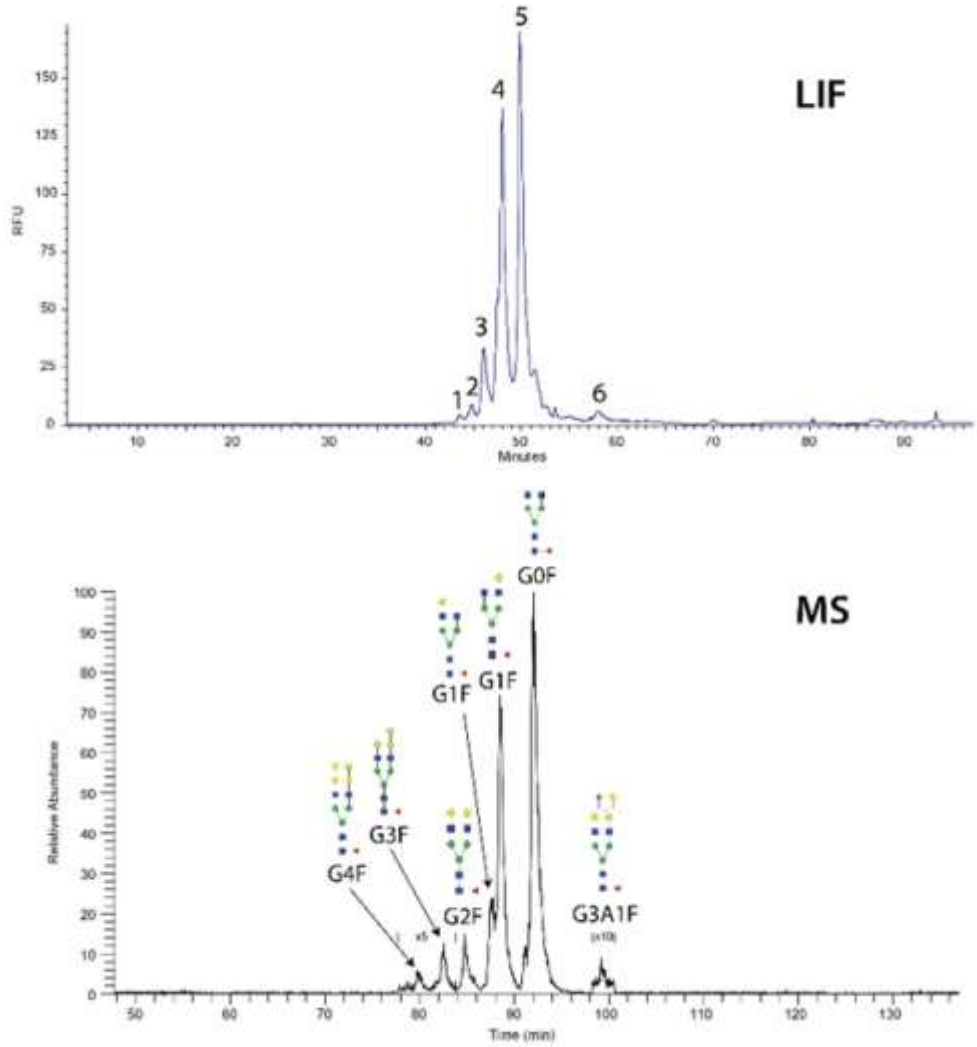
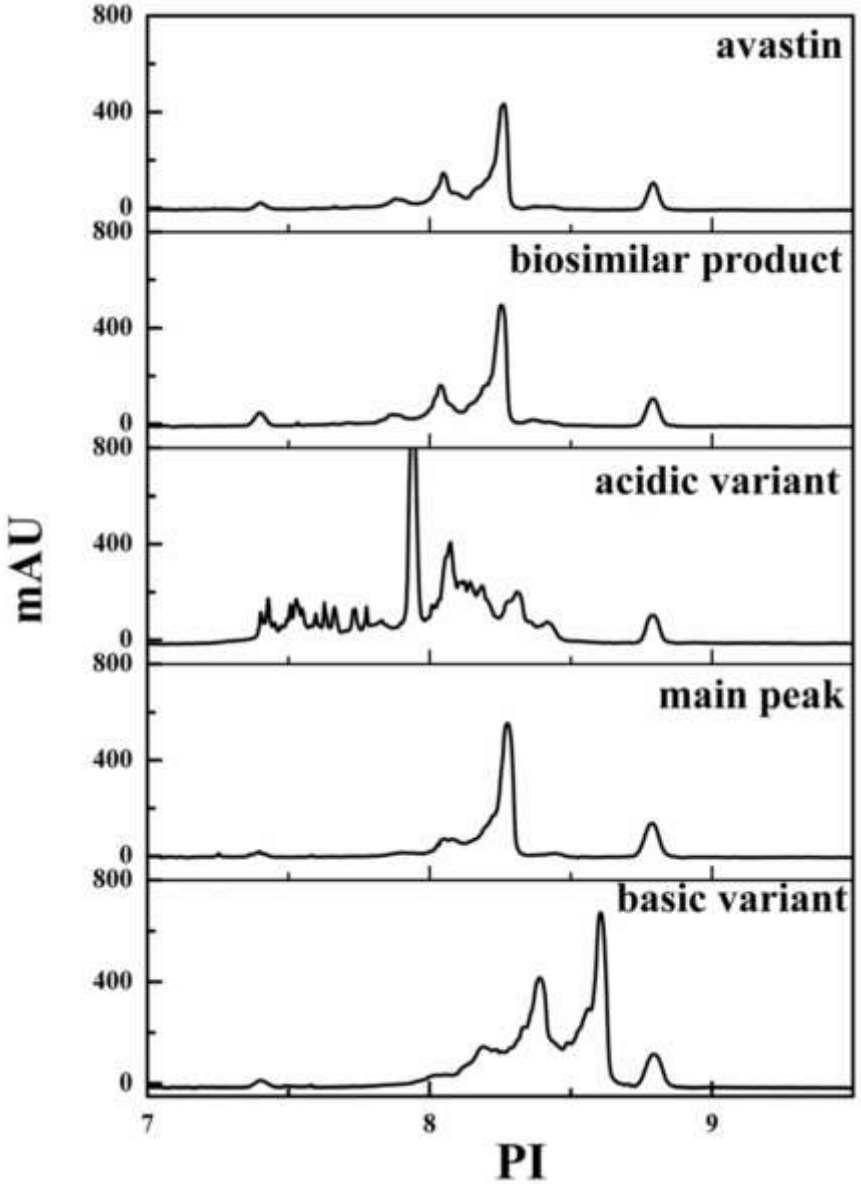


Fig. 2. Online CE-LIF-MS analysis of Teal™-labeled NISTmAb released *N*-glycans. LIF and MS base peak electropherogram (m/z 620-1,800) of Teal-NISTmAb glycans using ammonium hydroxide based BGE. Reprinted from [28]. Copyright (2017) Wiley.



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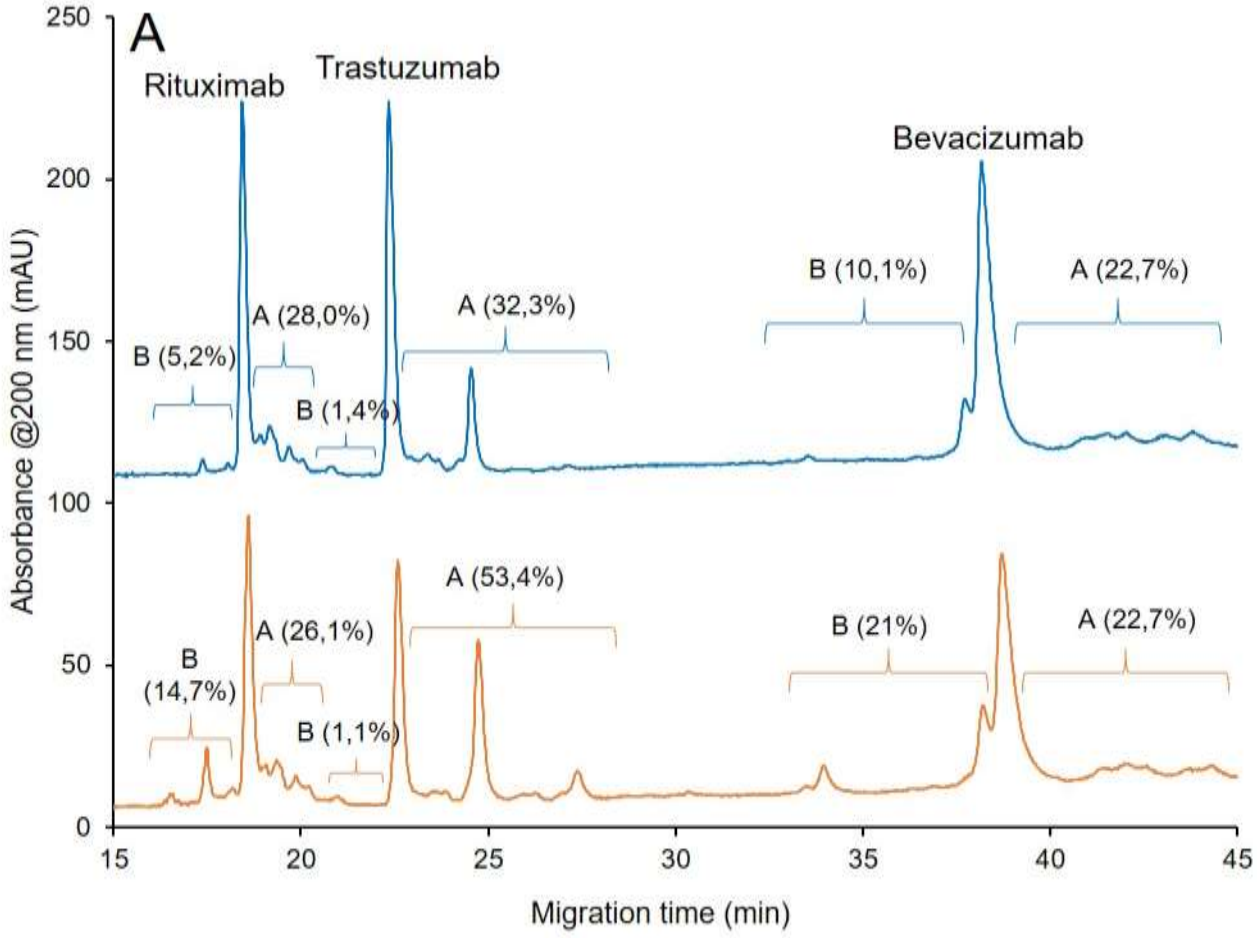

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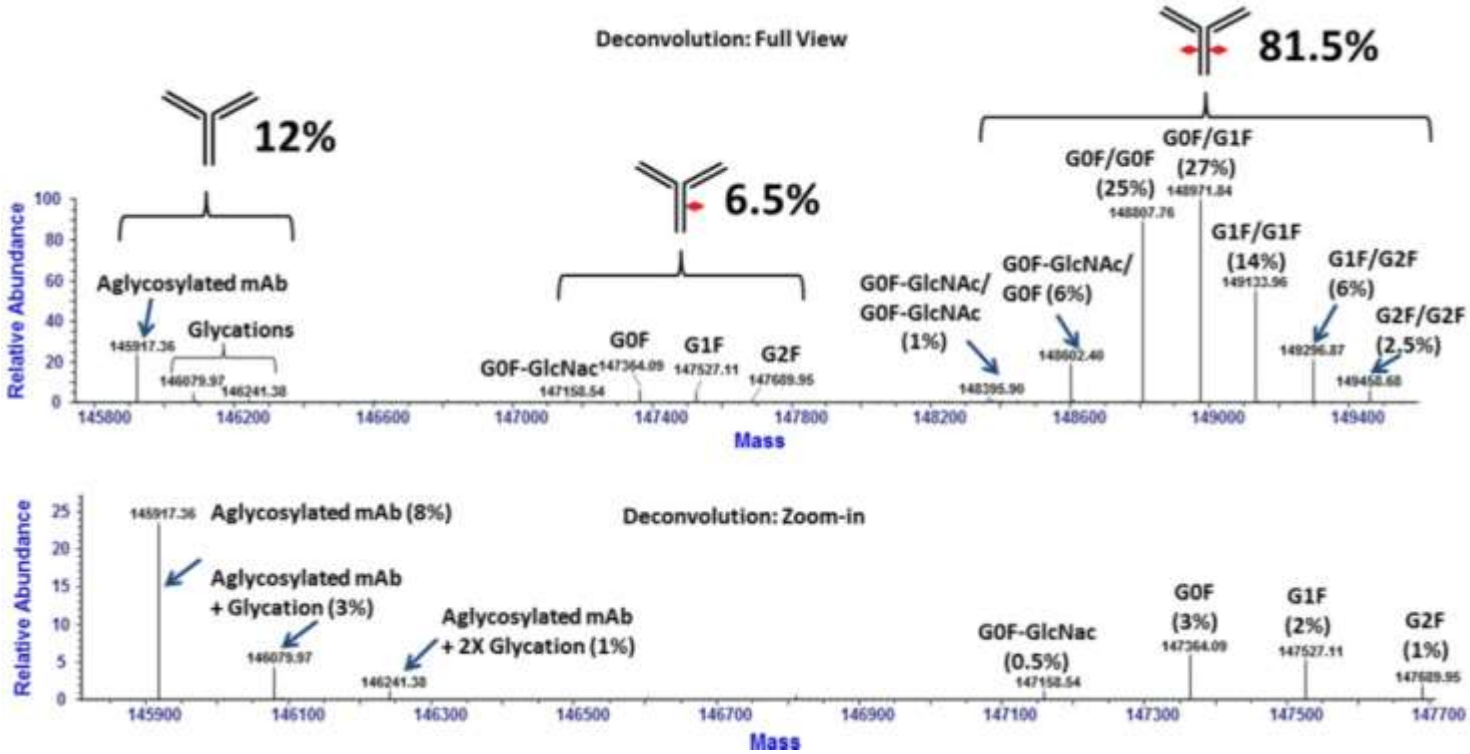

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